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HYSEQ, INC. [/]; (). BAIER, Joerg [/]; (). ABRAMS,  
Samuel, B.; ().

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(54) Title: POLYNUCLEOTIDE ARRAYS AND METHODS OF MAKING AND USING THE SAME

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## (57) Abstract

The present invention relates to methods of using labeled tracers to generate spatially addressable arrays of immobilized molecules, particularly polynucleotides, that can be normalized for differences in immobilization efficiencies at different addresses in the array. It also relates to the arrays generated by the method and to use of these arrays to enhance discrimination in array-based assays, particularly the discrimination between perfectly matched hybrids and hybrids containing a single mismatch in nucleic acid hybridization assays.

## (57) Abrégé

L'invention concerne des techniques permettant d'utiliser des indicateurs marqués, afin de produire des groupes de molécules immobilisées spatialement adressables, en particulier des polynucléotides pouvant être normalisés, de manière à présenter des différences dans l'efficacité de l'immobilisation au niveau d'adresses différentes dans le groupe. L'invention concerne également les groupes produits à l'aide de cette technique, et l'utilisation de ces groupes pour améliorer la discrimination dans des dosages à base de groupes, en particulier la discrimination entre des hybrides parfaitement appariés et des hybrides contenant un seul mésappariement dans des dosages d'hybridation d'acide nucléique.

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<b>(21) International Application Number:</b> PCT/US99/29194 <b>(22) International Filing Date:</b> 9 December 1999 (09.12.99)  <b>(30) Priority Data:</b> 60/111,961 11 December 1998 (11.12.98) US 09/454,822 6 December 1999 (06.12.99) US  <b>(71) Applicant:</b> HYSEQ, INC. [US/US]; 670 Almanor Avenue, Sunnyvale, CA 94086 (US).  <b>(72) Inventor:</b> BAIER, Joerg; 325 Catamaran Street, Foster City, CA 94404 (US).  <b>(74) Agents:</b> ABRAMS, Samuel, B. et al.; Pennic & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> POLYNUCLEOTIDE ARRAYS AND METHODS OF MAKING AND USING THE SAME  <b>(57) Abstract</b>  The present invention relates to methods of using labeled tracers to generate spatially addressable arrays of immobilized molecules, particularly polynucleotides, that can be normalized for differences in immobilization efficiencies at different addresses in the array. It also relates to the arrays generated by the method and to use of these arrays to enhance discrimination in array-based assays, particularly the discrimination between perfectly matched hybrids and hybrids containing a single mismatch in nucleic acid hybridization assays.		

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**Description**

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**POLYNUCLEOTIDE ARRAYS AND METHODS OF MAKING AND  
USING THE SAME**

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This application claims priority to the Provisional Patent Application entitled, "IMPROVED POLYNUCLEOTIDE ARRAYS AND METHODS OF MAKING AND USING THE SAME," Serial No. 60/111,961, filed December 11, 1998.

**1. FIELD OF THE INVENTION**

The present invention relates to spatially-addressable arrays of molecules, particularly biological molecules such as peptides and oligonucleotide probes, and methods of making and using the same.

**2. BACKGROUND OF THE INVENTION**

Recent advances in the ability to construct arrays of biological molecules has greatly facilitated the ease and speed with which certain biological assays can be performed. For example, in the areas of nucleic acid sequencing and analysis, the advent of new technologies for constructing arrays of immobilized target nucleic acids or oligonucleotide probes has enabled the rapid screening and sequencing of nucleic acids. Arrays of peptides and small biomolecules have also proven useful in binding assays used in pharmaceutical development. The usefulness of these arrays depends on the ability to generate arrays with spatially addressable regions of defined composition or sequence.

Several technologies have been developed for producing these arrays of biological molecules. Several researchers have devised methods for *in situ* synthesis of arrays of biological polymers, such as nucleic acids, peptides, and carbohydrates. These methods use, for example, physical barriers to separate regions, devices (such as inkjet printers) for precise delivery of reagents to regions, or masking techniques that allow the use of light to determine the course of synthesis. See, e.g., WO 90/03382; Fodor *et al.*, 1991, Science 251:767-73; Pease *et al.*, 1994, Proc. Natl. Acad. Sci. 91:5022-26; U.S. Patent No. 5,424,186, to Fodor *et al.* Alternatively, presynthesized biomolecules or biological polymers may be attached directly to the substrate at precise positions using a variety of techniques, ranging from simple spotting to robotic delivery systems. A variety of different substrates and techniques for attaching the biomolecules to the substrates are also available.

5 As noted above, arrays of nucleic acids have proven particularly valuable. The  
ability to perform many previously available techniques has been greatly enhanced by  
availability of arrays, which permit many assays to be performed simultaneously on a single  
10 array rather than having to do each assay individually. Other techniques that would have  
5 been virtually impossible are now possible using polynucleotide arrays.

One technique that has been particularly enhanced by the availability of arrays of  
nucleic acids is sequencing by hybridization (SBH). SBH is a technique for rapidly  
15 sequencing nucleic acids without using gels. In SBH, polynucleotides having overlapping  
sequences are hybridized to a target nucleic acid. The sequences of the polynucleotides that  
10 hybridize are then determined and the common sequences overlapped to generate the  
sequence of the nucleic acid. The use of arrays has allowed the generation of sufficient  
20 hybridization information to make SBH feasible on a large scale.

SBH is divided into three formats, depending on the nature of the array and the way  
in which it is interrogated. In Format I, the target nucleic acid is immobilized and the  
15 labeled polynucleotides are in solution. In Format II, the polynucleotides are immobilized  
and the labeled target nucleic acid is in solution. In Format III, immobilized  
25 polynucleotides are hybridized with an unlabeled target nucleic acid and labeled  
oligonucleotide probes. Hybridization is assayed by ligating the labeled oligonucleotide  
probes to the immobilized polynucleotides. All three formats require the ability to  
30 distinguish perfectly matched hybrids from hybrids that contain a single mismatch at any  
position. For a more detailed discussion of SBH and the three formats, see WO 98/31836,  
particularly at pages 1-3.

While the demand for biological arrays, and in particular polynucleotide arrays, is  
35 high, current methodologies for constructing such arrays still suffer from certain difficulties.  
25 The most common difficulty is assaying the quality and integrity of an array once it has  
been fabricated. While the chemistries involved in producing the arrays are relatively well  
understood, methods for synthesizing arrays still suffer from lack of reliability and  
40 reproducibility, and even failure. However, identifying regions of attachment failures is  
very difficult, particularly with the small spots found in miniaturized arrays. Thus, quality  
30 control of produced arrays is very difficult to maintain. Furthermore, even minor variations  
in attachment efficiencies can make interpretation of results generated from such arrays very  
difficult, as the researcher may not be able to tell whether a difference in signal is real or  
45 merely an artifact of the attachment process. This problem is particularly acute in  
applications such as sequencing by hybridization, which require extremely accurate  
50 differentiation of even minor differences in hybridization.

### 3. SUMMARY OF THE INVENTION

These and other shortcomings in the art are overcome by the present invention, which in one aspect provides spatially addressable arrays of immobilized molecules in which each spot in the array contains an amount of a detectable label which is proportional to the amount of molecule immobilized at that spot. The label can be any molecule which is capable of producing a detectable, quantifiable signal, such as a radioisotope, fluorophore, chromophore, chemiluminescent moiety, etc.. The labels at each spot may be the same or different, but are preferably the same.

In another aspect, the invention provides methods of making arrays of immobilized molecules in which each spot in the array contains an amount of a detectable label which is proportional to the amount of molecule immobilized at that spot. In the method, a molecule to be immobilized at a particular spot on the array is "spiked" with a detectable label capable of immobilizing to the substrate with the same efficiency as the molecule. The molecules to be immobilized at different spots are each "spiked" with the same proportion of label. Thus, following immobilization, each spot in the array contains an amount of label which is proportional to the efficiency of the immobilization technique. Following synthesis, the array can be scanned or otherwise analyzed for detectable signal to monitor the fidelity of the array synthesis.

In a preferred embodiment, the label is attached to, incorporated within, or otherwise associated with the same type of molecule as that to be immobilized. Accordingly, in this preferred embodiment of the methods, the molecule to be immobilized is spiked with a small amount of labeled molecule of the same type. Again, the molecules to be immobilized at different locations are each spiked with the same proportion of labeled molecule.

In another aspect, the invention provides methods of increasing the accuracy of array-based assays. In the method, background signals produced from an array of spatially addressable immobilized molecules according to the invention are quantified and recorded. The array is contacted with a target molecule capable of interacting with at least one of the immobilized molecules. The target molecule is labeled in some manner to produce an assay signal, or the interaction between the target and immobilized molecule is such that only those spots on the array where interaction has taken place produce a detectable assay signal. Following contact and optional washing, the array is scanned or otherwise analyzed for detectable assay signal, and the signal from each labeled spot quantified. The intensities of the signals from the respective spots are then normalized, typically by obtaining the ratio  $I_t/I_b$  (where  $I_t$  is the assay signal intensity and  $I_b$  is the background signal intensity), to

5 account for signal differences caused by deviations in the quantities of immobilized molecules. This normalization process permits signal intensities from different spots on the array to be directly compared, regardless of the fidelity of the array synthesis.

10 The labels giving rise to the background signals and assay signals, *i.e.*, the moieties  
5 used to label the array spots and target molecules, respectively, may be the same or different. In instances where the same label is used, the background signals should be recorded prior to contacting the array with the target molecule. The assay signal is then  
15 obtained by subtracting the background signal from a particular spot from the total signal from that spot. In this embodiment, the assay signals are normalized by obtaining the ratio  
10  $(I_a - I_b)/I_b$ , where  $I_a$  and  $I_b$  are as previously defined. When different labels are used, the background signal can be detected and recorded prior to, concomitant with, or after  
20 detection of assay signals.

The spatially-addressable arrays and methods of the invention provide myriad advantages over conventional arrays of immobilized molecules. Due to the presence of the  
15 labels, the quality of any array can be verified prior to use by simply scanning the array for detectable signal, even in instances where the array is highly miniaturized. Variability in the  
25 quantities of molecules immobilized at different positions within the array, and more importantly the complete absence of particular spots from the array, will be readily apparent. Moreover, because the quantity of label at each spot in the array correlates with  
30 the quantity of molecule immobilized at that spot, assay signals produced from different spots in the arrays can be normalized and directly compared, regardless of the fidelity of the array synthesis. This feature is particularly important for assays in which signal intensities are critical to determining whether the signal is real.

35 The advantages of the methods and arrays of the invention are illustrated by way of  
25 working examples involving nucleic acid hybridization assays. In the examples, hybridization assays performed with polynucleotide arrays according to the invention were  
40 able to discriminate perfectly complementary hybrids from mismatched hybrids which could not be discriminated using conventional polynucleotide arrays.

#### 30 4. BRIEF DESCRIPTION OF THE FIGURES

45 FIG. 1 shows a scan of a polynucleotide array produced with labeled tracer polynucleotides in each polynucleotide mixture. The scan detects the fluorescence at each spot.

50 FIG. 2 shows a scan of the array of FIG. 1 after hybridization and ligation with the  
35 target DNA and labeled polynucleotides.



## 5. DETAILED DESCRIPTION OF THE INVENTION

### 5.1 DEFINITIONS

As used herein, the following terms shall have the following meanings:

“Spatially addressable array” refers to an array in which each element or component of the array is identifiable by its spatial address, for example its xyz coordinates. Spatial addressable arrays according to the invention can be one dimensional, for example a linear array; two dimensional; or three dimensional.

“Address” or “spot” refers to a particular position in an array. Each address or spot has unique xyz coordinates. The structure of a compound immobilized at a particular address or spot is definable by its coordinates.

“Polynucleotide” refers to a nucleic acid sequence which is immobilized on a substrate. The polynucleotides of the present invention can contain as few as four bases or as many as several hundred or more bases. The polynucleotides can be composed of natural or modified bases or combinations thereof, and can contain one or more modified interlinkages.

“Target nucleic acid” refers to a nucleic acid of known or unknown sequence to be analyzed. The target nucleic acid can be virtually any number of nucleotides in length, but typically is longer than the polynucleotides of the array.

“Tracer moiety” refers to a molecule capable of generating a detectable signal (*i.e.*, a label or labeled molecule) that is or is capable of being immobilized on the spots of the array, and whose amount is proportional to the amount of a molecule of interest immobilized at that spot. The presence of the tracer moiety allows normalization of the array for differences in immobilization efficiencies.

### 5.2 THE INVENTION

The problems in the art discussed in the Background section are solved by the present invention. The methods of the present invention enable the quality and integrity of arrays of immobilized molecules to be simply and reliably assessed. Quality is assessed by the use of small quantities of labels, either by themselves or attached to the molecules of interest. The labels or labeled molecules are immobilized on the substrate along with the molecules of interest, and their immobilization is proportional to the immobilization of the molecules of interest. The intensity of the signal from the immobilized labels at a given location provides an assessment of the amount of the molecule of interest immobilized on the substrate at that location, and thus provides a way to verify the quality and integrity of the array as a whole. This intensity information is also useful when the array is

5 interrogated, as it provides a way to distinguish real differences in signal intensity due to experimental results from artifactual variations due simply to inconsistent immobilization of the molecules. The invention is also directed to arrays made by these methods and their use in various assay techniques.

10 5 The present methods are applicable to a wide variety of different molecules that may be placed in arrays. The methods are particularly exemplified herein in terms of polynucleotides immobilized on a substrate, but they are equally applicable to other types of molecules. For example, one of skill in the art could easily adapt the present methods to apply to other nucleic acids (both DNA and RNA), peptides, polypeptides, proteins, 15 carbohydrates, small biomolecules (e.g. drug candidates), or any other type of molecule that can be immobilized on a substrate by any method. Preferably, the molecule is one that can be labeled, although this is not necessary if a label can be immobilized on the substrate in a fashion similar to the molecule.

20 The arrays of the present invention may be of any desired size, from two spots to  $10^6$  spots or even more. The upper and lower limits on the size of the substrate are determined solely by the practical considerations of working with extremely small or large substrates. For a given substrate size, the upper limit is determined only by the ability to create and detect the spots in the array. The preferred number of spots on an array generally depends on the particular use to which the array is to be put. For example, sequencing by 25 hybridization will generally require large arrays, while mutation detection may require only a small array. In general, preferred arrays contain from 2 to about  $10^6$  spots, more preferably from about 100 to about  $10^5$  spots, particularly preferably from about 400 to about  $10^4$  spots, and most preferably between about 500 and about 2000 spots.

30 Furthermore, not all spots on the array need be unique. Indeed, in many applications, 25 redundancies in the spots are desirable for the purposes of acting as internal controls.

40 A variety of techniques have been described for synthesizing and/or immobilizing arrays of polynucleotides, including *in situ* synthesis, where the polynucleotides are synthesized directly on the surface of the substrate (see, e.g., U.S. Patent No. 5,744,305 to Fodor, et al.,) and attachment of pre-synthesized polynucleotides to the surface of a 30 substrate at discrete locations (see, e.g., WO 98/31836). Additional methods are described in WO 98/31836 at pages 41-45 and 47-48, among other places. The present invention is 45 suitable for use with any of these currently available, or later developed, techniques.

50 In embodiments involving immobilization of pre-synthesized polynucleotides, the polynucleotide reagent to be deposited at a particular spot contains a small quantity, 35 typically 0.01 to 0.15%, and preferably 0.08%, of a label, typically a labeled polynucleotide.

5 The polynucleotide reagent is then deposited on the substrate at a spatially defined region,  
i.e., at a particular spot. After immobilization, the spot contains an amount of labeled  
polynucleotide which is proportional to the amount of polynucleotide immobilized at that  
10 spot. Depositing a number of such polynucleotide reagents at different spatial addresses  
5 yields an array of polynucleotides whose sequences are identifiable by their spatial  
addresses. Moreover, each spot in the array contains an amount of labeled polynucleotide  
that is proportional to the amount of polynucleotide immobilized at that spot.

15 In embodiments involving *in situ* synthesis of polynucleotides, the polynucleotides  
are synthesized in their usual manner. At the synthetic step which adds the last nucleotide,  
10 the nucleoside phosphoramidite reagent to be deposited contains a small quantity, typically  
0.01 to 0.15%, and preferably 0.08%, of a label, typically a labeled nucleoside  
20 phosphoramidite. The synthetic scheme yields an array of polynucleotides whose  
sequences are identifiable by their spatial addresses. Moreover each spot in the array  
contains an amount of labeled polynucleotide that is proportional to the amount of full-  
15 length polynucleotide synthesized at that spot.

25 While the above method contemplates labeling the last nucleotide of the  
polynucleotide, those of skill in the art will appreciate that other positions, or additional  
positions, could be similarly labeled to provide information about the proportions of  
truncated polynucleotides synthesized. In these embodiments, the labels used at the various  
30 steps should be distinguishable from one another.

Moreover, while the *in situ* synthesis method is described utilizing phosphoramidite  
reagents, it will be recognized that other reagents utilizing other synthesis strategies can also  
35 be employed, and in certain circumstances may be preferable, depending on the stability of  
the chosen label to the synthesis conditions. Non-limiting examples of suitable chemistries  
25 and reagents are described, for example in *Oligonucleotide Synthesis: A Practical  
Approach*, M.J. Gait, Ed., IRL Press, Oxford, England, 1985.

40 The members of the arrays of the invention are immobilized on a solid substrate.  
The nature and geometry of the solid substrate will depend upon a variety of factors,  
including, among others, the type of array (e.g., one-dimensional, two-dimensional or three-  
30 dimensional) and the mode of attachment (e.g., covalent or non-covalent). Generally, the  
45 substrate can be composed of any material which will permit immobilization of the  
polynucleotide and which will not melt or otherwise substantially degrade under the  
conditions used to hybridize and/or denature nucleic acids. In addition, where covalent  
immobilization is contemplated, the substrate should be activatable with reactive groups  
50 35 capable of forming a covalent bond with the polynucleotide to be immobilized.

5 A number of materials suitable for use as substrates in the instant invention have been described in the art. Exemplary suitable materials include, for example, acrylic, styrene-methyl methacrylate copolymers, ethylacrylic acid, acrylonitrile-butadiene-  
10 styrene (ABS), ABS/polycarbonate, ABS/polysulfone, ABS/polyvinyl chloride, ethylene  
5 propylene, ethylene vinyl acetate (EVA), nitrocellulose, nylons (including nylon 6, nylon 6/6, nylon 6/6-6, nylon 6/9, nylon 6/10, nylon 6/12, nylon 11 and nylon 12), polycrylonitrile (PAN), polyacrylate, polycarbonate, polybutylene terephthalate (PBT),  
15 polyethylene terephthalate (PET), polyethylene (including low density, linear low density, high density, cross-linked and ultra-high molecular weight grades), polypropylene  
10 homopolymer, polypropylene copolymers, polystyrene (including general purpose and high impact grades), polytetrafluoroethylene (PTFE), fluorinated ethylene-propylene (FEP),  
20 ethylene-tetrafluoroethylene (ETFE), perfluoroalkoxyethylene (PFA), polyvinyl fluoride (PVF), polyvinylidene fluoride (PVDF), polychlorotrifluoroethylene (PCTFE),  
polyethylene-chlorotrifluoroethylene (ECTFE), polyvinyl alcohol (PVA), silicon styrene-  
15 acrylonitrile (SAN), styrene maleic anhydride (SMA), metal oxides and glass.

25 The substrate may be in the form of beads, particles or sheets, and may be permeable or impermeable, depending on the type of array. For example, for linear or three-dimensional arrays the substrate may consist of bead or particles (such as conventional solid phase synthesis supports), fibers (such as glass wool or other glass or plastic fibers) or glass  
30 or plastic capillary tubes. For two-dimensional arrays, the substrate is preferably in the form of plastic or glass sheets in which at least one surface is substantially flat. Particularly preferred substrates for use with two-dimensional arrays are glass slides.

35 The composition of the immobilized polynucleotides is not critical. The only requirement is that they be capable of hybridizing to a target nucleic acid of complementary  
25 sequence. For example, the polynucleotides may be composed of all natural or all synthetic nucleotide bases, or a combination of both. Non-limiting examples of modified bases  
40 suitable for use with the instant invention are described, for example, in Practical Handbook of Biochemistry and Molecular Biology, G. Fasman, Ed., CRC Press, 1989, pp. 385-392.

While in most instances the polynucleotides will be composed entirely of the natural bases  
30 (A, C, G, T or U), in certain circumstances the use of synthetic bases may be preferred.

45 Moreover, while the backbones of the polynucleotides will typically be composed entirely of "native" phosphodiester linkages, they may contain one or more modified linkages, such as one or more phosphorothioate, phosphoramidite or other modified linkages. As a specific example, one or more immobilized polynucleotides may be a  
50 35 peptide nucleic acid (PNA), which contains amide interlinkages. Additional examples of

5 modified bases and backbones that can be used in conjunction with the invention, as well as  
methods for their synthesis can be found, for example, in Uhlman & Peyman, 1990,  
Chemical Review 90(4):544-584; Goodchild, 1990, Bioconjugate Chem. 1(3):165-186;  
10 Egholm et al., 1992, J. Am. Chem. Soc. 114:1895-1897; Gryaznov et al., J. Am. Chem. Soc.  
5 116:3143-3144, as well as the references cited in all of the above.

While the immobilized polynucleotides will in most instances be a contiguous  
stretch of nucleotides, they need not be. Stretches of nucleotides can be interrupted by one  
15 or more linker molecules that do not participate in sequence-specific base pairing  
interactions with a target nucleic acid. The linker molecules may be flexible, semi-rigid or  
10 rigid, depending on the desired application. A variety of linker molecules useful for spacing  
one molecule from another or from a solid surface have been described in the art (and are  
20 described more thoroughly infra); all of these linker molecules can be used to space regions  
of immobilized polynucleotides from one another. In a preferred embodiment of this aspect  
of the invention, the linker moiety is from one to ten, preferably one to six, alkylene glycol  
15 moieties, preferably ethylene glycol moieties.

The immobilized polynucleotides may be as few as four, or as many as hundreds, or  
even more, nucleotides in length. Specifically contemplated as polynucleotides according to  
the invention are nucleic acids that are typically referred to in the art as oligonucleotides and  
also those referred to as nucleic acids. Thus, the arrays of the present invention are useful  
30 20 not only in applications where target nucleic acids are hybridized to immobilized arrays of  
relatively short (i.e., 6-20 nucleotide) probes (such as format II SBH), but also in  
applications where relatively short probes are hybridized to arrays of immobilized nucleic  
acids.

35 The polynucleotides of the array can be of any desired sequence. In a preferred  
25 embodiment, they can comprise all possible polynucleotides of a given length N, which  
would result in an array of  $4^N$  unique elements. For all polynucleotides of, for example, 6  
bases in length, the sequences would comprise an array with 4096 unique elements.  
40 Alternatively, the polynucleotides can make up the "universal set" for sequencing a nucleic  
acid, as discussed in WO 98/31836, particularly pages 27-29.

30 In an alternative embodiment, the set of polynucleotides may correspond to  
45 particular mutations that are to be identified in a known sequence. For example, if a  
particular nucleic acid is known to contain an unidentified mutation at a particular position,  
then the mutated position can be identified with an array of eight polynucleotides, three  
corresponding to the three possible substitutions at that position, one corresponding to the  
50 35 deletion of the base at that position, and four corresponding to the insertion of the four

possible bases at that position. Alternatively, for a known gene that may contain any of several possible identified mutations, the array can comprise polynucleotides corresponding to the different possible mutations. This embodiment is particularly useful for genes like oncogenes and tumor suppressors, which frequently have a variety of known mutations in different positions. Using arrays facilitates determining whether or not these genes contain mutations by allowing simultaneous screening with polynucleotides corresponding to each of these different positions.

In another alternative embodiment, each spot of the array can comprise a mixture of polynucleotides of different sequences. These mixtures may comprise degenerate polynucleotides of the structure  $N_xB_yN_z$ , wherein N represents any of the four bases and varies for the polynucleotides in a given mixtures, B represents any of the four bases but is the same for each of the polynucleotides in a given mixture, and x, y, and z are integers. Arrays comprising this type of mixture are useful in, for example, sequencing by hybridization. Alternatively, the spots may comprise mixtures of polynucleotides that correspond to different regions of a known nucleic acid; these regions may be overlapping, adjacent, or nonadjacent. Arrays comprising these types of mixtures are useful in, for example, identifying specific nucleic acids, including those from particular pathogens or other organisms. Both types of mixtures are discussed in WO 98/31836, particularly at pages 123-128.

The polynucleotides can be isolated from biological samples, generated by PCR reactions or other template-specific reactions, or made synthetically. Methods for isolating polynucleotides from biological samples and/or PCR reactions are well-known in the art, as are methods for synthesizing and purifying synthetic polynucleotides. Probes isolated from biological samples and/or PCR reactions may, depending on the desired mode of immobilization, require modification at the 3'- or 5'-terminus, or at one or more bases, as will be discussed more thoroughly below. Moreover, since the polynucleotide must be capable of hybridizing to a target nucleic acid, if not already single stranded, it should preferably be rendered single stranded, either before or after immobilization on the substrate.

The polynucleotides can be immobilized on the substrate using a wide variety of techniques. For example, the polynucleotides can be adsorbed or otherwise non-covalently associated with the substrate (for example, immobilization to nylon or nitrocellulose filters using standard techniques); they may be covalently attached to the substrate; or their association may be mediated by specific binding pairs, such as biotin and streptavidin. Of these methods, covalent attachment is preferred.

5 In order to effect covalent attachment, the substrate must first be activated, *i.e.*,  
treated so as to create reactive groups on or within the substrate that can react with a  
reactive group on the polynucleotide to form a covalent linkage. Those of skill in the art  
10 will recognize that the desired reactive group will depend on the chemistry used to attach  
5 the polynucleotides to the substrate and the composition of the substrate. Typical reactive  
groups useful for effecting covalent attachment of polynucleotides to substrates include  
hydroxyl, sulfonyl, amino, epoxy, isothiocyanate and carboxyl groups; however, other  
15 reactive groups as will be apparent to those having skill may also be used and are also  
included within the scope of the invention.

10 For a review of the myriad techniques that can be used to activate the substrates with  
suitable reactive groups, *see* Wiley Encyclopedia of Packaging Technology, 2d Ed., Brody  
& Marsh, Ed., "Surface Treatment," pp. 867-874, John Wiley & Sons (1997), and the  
20 references cited therein (hereinafter "Surface Treatment"). Chemical methods suitable for  
generating amino groups on silicon oxide substrates are described in Atkinson & Smith,  
15 "Solid Phase Synthesis of Oligodeoxyribonucleotides by the Phosphite Triester Method,"  
25 *In: Oligonucleotide Synthesis: A Practical Approach*, M J Gait, Ed., 1984, IRL Press,  
Oxford, particularly at pp. 45-49 (and the references cited therein); chemical methods  
suitable for generating hydroxyl groups on silicon oxide substrates are described in Pease et  
30 al., 1994, *Proc. Natl. Acad. Sci. USA* 91:5022-5026 (and the references cited therein);  
20 chemical methods for generating functional groups on polymers such as polystyrene,  
polyamides and grafted polystyrenes are described in Lloyd-Williams *et al.*, 1997, *Chemical  
Approaches to the Synthesis of Peptides and Proteins*, Chapter 2, CRC Press, Boca Raton,  
35 FL (and the references cited therein).

Those of skill in the art will recognize that in embodiments employing covalent  
25 attachment, the covalent bond formed between the polynucleotide and the substrate must be  
substantially stable to the various conditions under which the array will be assayed, to avoid  
40 loss of polynucleotide during the assay. One such stable bond is the phosphodiester bond,  
which connects the various nucleotides in a polynucleotide, and which can be conveniently  
formed using well-known chemistries (*see, e.g.*, *Oligonucleotide Synthesis: A Practical  
30 Approach*, 1984, *supra*). Other stable bonds suitable for use with hydroxyl-activated  
45 substrates include phosphorothioate, phosphoramidite, or other modified nucleic acid  
interlinkages. For substrates modified with amino groups, the bond could be a  
phosphoramidate, amide or peptide bond. When substrates are activated with epoxy  
functional groups, a stable C-N bond could be formed. Suitable reagents and conditions for  
50

5 forming such stable bonds are well known in the art. Other stable bonds suitable for use with the arrays of the invention will be apparent to those of skill in the art.

10 In embodiments in which pre-synthesized polynucleotides are covalently attached to the substrate, the polynucleotides may be *via* their 3'-terminus, 5'-terminus or by way of a  
5 reactive group at one of the bases. Synthesis supports and synthesis reagents useful for modifying the 3'- and/or 5'-terminus of synthetic polynucleotides, or for incorporating a base modified with a reactive group into a synthetic polynucleotide, are well-known in the  
15 art and are also commercially available.

For example, methods for synthesizing 5'-modified polynucleotides are described in  
10 Agarwal et al., 1986, Nucl. Acids Res. 14:6227-6245 and Connelly, 1987, Nucl. Acids Res. 15:3131-3139. Commercially available products for synthesizing 5'-amino modified  
20 polynucleotides include the N-TFA-C6-AminoModifier™, N-MMT-C6-AminoModifier™ and N-MMT-C12-AminoModifier™ reagents available from Clontech Laboratories, Inc., Palo Alto, California.

15 Methods for synthesizing 3'-modified polynucleotides are described in Nelson et al., 1989, Nucl. Acids Res. 17:7179-7186 and Nelson et al., 1989, Nucl. Acids Res. 17:7187-7194. Commercial products for synthesizing 3'-modified polynucleotides include the 3'-Amino-ONT™ controlled pore glass and Amino Modifier II™ reagents available from  
25 Clontech Laboratories, Inc., Palo Alto, California.

30 Other methods for modifying the 3' and/or 5' termini of polynucleotides, as well as for synthesizing polynucleotides containing appropriately modified bases are provided in Goodchild, 1990, Bioconjugate Chem. 1:165-186, and the references cited therein. Chemistries for attaching such modified polynucleotides to substrates activated with  
35 appropriate reactive groups are well-known in the art (*see, e.g.*, Ghosh & Musso, 1987, Nucl. Acids Res. 15:5353-5372; Lund et al., 1988, Nucl. Acids Res. 16:10861-10880; Rasmussen et al., 1991, Anal. Chem. 198:138-142; Kato & Ikada, 1996, Biotechnology and Bioengineering 51:581-590; T...ofeev et al., 1996, Nucl. Acids Res. 24:3142-3148;  
40 O'Donnell et al., 1997, Anal. Chem. 69:2438-2443).

Methods and reagents for modifying the ends of polynucleotides isolated from  
30 biological samples and/or for incorporating bases modified with reactive groups into nascent polynucleotides are also well-known and commercially available. For example, an isolated polynucleotide can be phosphorylated at the 5'-terminus with phosphorokinase and this phosphorylated polynucleotide covalently attached to an amino-activated substrate  
45 through a phosphoramidate or phosphodiester linkage. Other methods will be apparent to  
50 those of skill in the art.



5 In one convenient embodiment, pre-synthesized polynucleotides, modified at their  
3'- or 5'-termini with a primary amino group, are conjugated to a carboxy-activated  
substrate. Chemistries suitable for forming carbamate linkages between carboxyl and  
10 amino functional groups are well-known in the art of peptide chemistry (*see, e.g.*, Atherton  
& Sheppard, Knorr et al., 1989, Tet. Lett. 30(15):1927-1930; Bannworth & Knorr, 1991,  
Tet. Lett. 32(9):1157-1160; and Wilchek et al., 1994, Bioconjugate Chem. 5(5):491-492;  
15 Solid Phase Peptide Synthesis, 1989, IRL Press, Oxford, England and Lloyd-Williams et  
al., Chemical Approaches to the Synthesis of Peptides and Proteins, 1997, CRC Press, Boca  
Raton, FL and the references cited therein). Any of these methods can be used to conjugate  
20 amino-modified polynucleotides to a carboxy-activated substrate.

In another convenient embodiment, the polynucleotides are synthesized directly on a  
20 hydroxy-activated substrate using commercially available phosphoramidites synthesis  
reagents. In this mode, the polynucleotides are covalently attached to the substrate via their  
3'-termini by way of a phosphodiester linkage. Alternatively, photoprotected  
25 phosphoramidites and the photolithographic methods described in U.S. Patent No.  
5,744,305 to Fodor *et al.* and Pease *et al.*, 1994, *supra*, can be used.

Whether synthesized directly on the activated substrate or immobilized on the  
activated substrate after synthesis or isolation, the polynucleotides can optionally be spaced  
away from the substrate by way of one or more linkers. As will be appreciated by those  
30 having skill in the art, such linkers will be at least bifunctional, *i.e.*, they will have one  
functional group or moiety capable of forming a linkage with the activated substrate and  
another functional group or moiety capable of forming a linkage with another linker  
molecule or the polynucleotides. The linkers may be long or short, flexible or rigid,  
35 charged or uncharged, hydrophobic or hydrophilic, depending on the particular application.

25 In certain circumstances, such linkers can be used to "convert" one functional group  
into another. For example, an amino-activated substrate can be converted into a hydroxyl-  
activated substrate by reaction with, for example, 3-hydroxy-propionic acid. In this way,  
40 substrate materials which cannot be readily activated with a specified reactive functional  
group can be conveniently converted into an appropriately activated substrate. Chemistries  
30 and reagents suitable for "converting" such reactive groups are well-known, and will be  
apparent to those having skill in the art.

45 Linkers can also be used, where necessary, to increase or "amplify" the number of  
reactive groups on the activated substrate. For this embodiment, the linker will have three  
or more functional groups. Following attachment to the activated substrate by way of one  
50 35 of the functional groups, the remaining two or more groups are available for attachment of

5 polynucleotides. Amplifying the number of functional groups on the activated substrate in this manner is particularly convenient when the substrate cannot be readily activated with a sufficient number of reactive groups.

10 Reagents for amplifying the number of reactive groups are well-known and will be apparent to those of skill in the art. A particularly convenient class of amplifying reagents are the multifunctional epoxides sold under the trade name DENACOL™ (Nagassi Kasei Kogyo K.K.). These epoxides contain as many as four, five, or even more epoxy groups, and can be used to amplify substrates activated with reactive groups that react with epoxides, including, for example, hydroxyl, amino and sulfonyl activated substrates. The 15 resulting epoxy-activated substrate can be conveniently converted to a hydroxyl-activated substrate, a carboxy-activated substrate, or other activated substrate by well-known methods.

20 Linkers suitable for spacing biological molecules such as polynucleotides from solid surfaces are well-known in the art, and include, by way of example and not limitation, polypeptides such as polyproline or polyalanine, saturated or unsaturated bifunctional hydrocarbons such as 1-amino-hexanoic acid, polymers such as polyethylene glycol, etc. 25 1,4-Dimethoxytrityl-polyethylene glycol phosphoramidites useful for forming phosphodiester linkages with hydroxyl groups, as well as methods for their use in nucleic acid synthesis on solid substrates, are described, for example in Zhang et al., 1991, Nucl. Acids Res. 19:3929-3933 and Durand et al., 1990, Nucl. Acids Res. 18:6353-6359. Other useful linkers are commercially available.

30 A critical feature of the arrays of the invention is the presence of an amount of a label at each position within the array that is proportional to the amount of polynucleotide immobilized at that particular spot. Thus, it is important that the efficiencies of the coupling reactions which are used to immobilize the label and polynucleotide are substantially similar. For covalent attachment, this can be conveniently achieved by using the same immobilization reactive group on both the label and the polynucleotide.

35 For embodiments employing immobilization of pre-synthesized polynucleotides, a preferred label is a labeled polynucleotide. The primary sequences of the labeled and unlabeled polynucleotides at a particular spot may be the same or different. In fact, the same labeled polynucleotide may be used at each spot in the array. The only requirement is that the polynucleotide reagents deposited at each spot in the array be "spiked" with substantially the same proportion of labeled polynucleotide.

40 In a preferred embodiment, the same mixture of labeled polynucleotides is used to spike the polynucleotide reagent deposited at each spot. Using the same mixture of labeled

5 polynucleotides at each spot ensures that the labels at different spots do not induce  
sequence-specific anomalies in hybridization assays, *i.e.*, it ensures that the labels at each  
array spot interact similarly with a target nucleic acid in hybridization assays. Moreover,  
10 use of the same label at each spot reduces the number of labeled polynucleotides that need  
5 to be prepared. A particularly preferred mixture of ten labeled polynucleotides is described  
in the examples section.

Virtually any label that produces a detectable, quantifiable signal and that is capable  
15 of being immobilized on a substrate or attached to a polynucleotide can be used in  
conjunction with the arrays of the invention. Suitable labels include, by way of example  
10 and not limitation, radioisotopes, fluorophores, chromophores, chemiluminescent moieties,  
etc. In embodiments where the label is attached to a polynucleotide, the label can be  
20 attached to any part of the polynucleotide, including the free terminus or one or more of the  
bases. Preferably, the position of the label will not interfere with hybridization, detection or  
other post-hybridization modifications of the labeled polynucleotide. Suitable methods of  
15 making labeled polynucleotides are well known in the art.

25 Due to their ease of detection, polynucleotides labeled with fluorophores are  
preferred. Fluorophores suitable for labeling polynucleotides are described, for example, in  
the Molecular Probes catalog (Molecular Probes, Inc., Eugene OR 97402-9144), and the  
references cited therein. Methods for attaching fluorophore labels to polynucleotides are  
30 well known, and can be found, for example in Goodchild, 1990, *supra*. A preferred  
20 fluorophore label is the carboxylic acid of tetramethyl rhodamine (TAMRA dye), which is  
available from Molecular Probes.

35 In embodiments employing *in situ* synthesis, a preferred label is a fluorescently-  
labeled nucleic acid synthesis reagent, such as a labeled nucleoside phosphoramidite. The  
25 position at which the fluorophore is attached to the nucleoside phosphoramidite will depend  
on whether the label will be added at the terminal or internal nucleotides of the nascent  
40 polynucleotides. When a terminal label is desired, the fluorophore can be conveniently  
attached to the 5'-hydroxyl. When internal labels are desired, the fluorophore is preferably  
attached to the base, optionally by way of a linker. Methods suitable for making  
30 fluorescently-labeled phosphoramidite synthesis reagents are well-known in the art, and are  
described, for example, in Goodchild, 1990, *supra*.  
45

The amount of label used to "spike" the polynucleotide reagent to be deposited at a  
particular spot is not critical for success. However, the amount used should be sufficient to  
produce a detectable signal which does not result in a loss of dynamic range when the array  
50 is used in an assay. For the preferred polynucleotide arrays of the invention, which are  
35

5 synthesized by depositing pre-synthesized polynucleotides at discrete spots, it has been  
found that spiking the polynucleotide reagent with about 0.01 to 0.15%, preferably about  
0.08%, of a fluorescently-labeled polynucleotide yields good results. When mixtures of  
10 fluorescently-labeled polynucleotides are used, the total quantity of labeled polynucleotides  
5 used to spike the reagent should fall within the above-described ranges.

The polynucleotide arrays according to the invention can be used in virtually any  
array in which hybridization is desirable. For example, the polynucleotide arrays of the  
15 invention are useful for all three formats of sequencing by hybridization, as well as the  
myriad other hybridization arrays performed with arrays of nucleic acids and/or  
10 oligonucleotide probes described in the art.

For use in a hybridization array, the background signals from a polynucleotide array  
20 according to the invention are quantified and recorded. The mode of detection will depend  
on the nature of the label. For fluorescent labels, the background signals can be  
conveniently quantified by scanning the array with a confocal camera or with a CCD  
15 camera, as is well-known in the art.

Use of the arrays of the present invention contemplates the use of either probe  
25 polynucleotides or target nucleic acids that are capable of generating a signal when  
appropriately hybridized to the array. The probe polynucleotides or target nucleic acids  
may be labeled, for example, by the labels and techniques described *supra* for labeling the  
30 tracer polynucleotide. Alternatively, they may be labeled by any other technique known in  
the art. Preferred techniques include direct chemical labeling methods and enzymatic  
labeling methods, such as kinasing and nick-translation.

The array is contacted with a target nucleic acid, which may be labeled or unlabeled,  
35 depending on the particular array (for example, format II vs. format III SBH), under  
25 conditions which discriminate between perfectly complementary hybrids and hybrids  
containing one or more mismatches. The actual hybridization conditions used will depend  
upon, among other factors, the G+C content of the sequence of interest and the lengths of  
40 the immobilized polynucleotides comprising the array. Hybridization conditions useful for  
discriminating between perfect compliments and mismatches for a variety of hybridization  
30 arrays have been described in the art. For example, hybridization conditions useful for  
discriminating complementary and mismatched hybrids in a variety of SBH and other  
45 applications are described in U.S. Patent No. 5,525,464 to Drmanac *et al.*, WO 95/09248  
and WO 98/31836. A particularly detailed discussion of the theoretical and practical  
considerations involved in determining hybridization conditions, and including a discussion  
50 of the advantages of low-temperature washing steps, may be found in WO 98/31836,

5 particularly pages 50-62. Additional guidance may be found in Harnes and Higgins, Nucleic Acid Hybridization: A Practical Approach, 1985, IRL Press, Oxford, England.

10 Following contact, the array is optionally washed, typically under moderate- to high stringency conditions to remove unhybridized target. If the target is labeled, the array can  
5 be scanned or otherwise analyzed for detectable assay signal, and the signal from each labeled spot, or alternatively from all spots, quantified. Only those spots where hybridization occurred will produce a detectable assay signal. If each spot in array contains  
15 the same quantity of immobilized polynucleotide, in theory, the intensity of the assay signal at each spot will be proportional to the extent of hybridization at that spot. For example,  
10 spots containing perfectly complementary hybrids are expected to produce more intense assay signals than spots containing mismatched hybrids. In practice, however, differences  
20 in signal intensities between different spots may instead be due to differences in the amounts of polynucleotide immobilized at the respective spots.

25 Because each spot in the arrays of the invention contain an amount of a label or "tracer" proportional to the amount of polynucleotide immobilized at the particular spot, the assay signals obtained from the arrays of the invention can be normalized. As a  
30 consequence, signal intensities from spots within a single array, or across multiple arrays, can be directly compared, without regard to the fidelity of the particular array synthesis.

35 The method by which the signals are normalized will depend upon whether the  
30 tracer or background signals are the same as the assay signals, such as where the polynucleotides and target nucleic acid are labeled with the same fluorophore. In this embodiment, a normalized signal of a particular spot is defined by  $(I_a - I_b)/I_b$ , where  $I_a$  is the  
40 intensity of the assay signal of the spot (*i.e.*, intensity of the spot after hybridization) and  $I_b$  is the intensity of the background signal of the spot (*i.e.*, the intensity of the spot before  
25 hybridization).

45 In embodiments where the background and assay signals are different, *i.e.*, where the array spots and target nucleic acid are labeled with different fluorophores, the normalized signal for a spot is described by  $I_a/I_b$ , where  $I_a$  is the intensity of the assay signal  
40 of the spot and  $I_b$  is the intensity of the background signal of the same spot.

50 While the array is illustrated utilizing a labeled target nucleic acid, those of skill in  
30 the art will recognize that the arrays of the invention are also useful in assays employing unlabeled target nucleic acids, such as assays employing the principles of format III SBH. The only requirement is that some component of the particular assay generate a detectable  
35 signal at spots where hybridization occurs.

5                   6.     **EXAMPLE: TRACER LABELING OF A POLYNUCLEOTIDE ARRAY**

          An array of polynucleotides was generated using the surface of a glass slide as the substrate. The polynucleotides used were 8 bases long, with an information content corresponding to all possible 5-base sequences. The polynucleotides all had the structure

10                   5       5'-36C(spacer)-NNNBBBBB-3',

          where:

          36C(spacer) is a standard, commercially available 36 carbon spacer element;

15                   N represents a degenerate position generated by synthesizing the polynucleotide with an equimolar mixture of all four bases according to standard methods (*i.e.*, each location on the array contained a mixture of polynucleotides degenerate at the N positions); and

20                   B represents any one of the four bases (*i.e.*, each location on the array contained a mixture of polynucleotides identical at the B positions).

          For the methods of the invention, a mixture of 10 labeled polynucleotide mixtures was prepared. The polynucleotides in the 10 mixtures had the structure describe above, and the labeled mixture consisted of the following sequences:

                  NNNGGCAT    NNNCGGAG    NNNAACTG    NNNATGAA    NNNTGTAC  
30                   20   NNNACTGG    NNNGAACC    NNNTACAG    NNNCTGGA    NNNCCGGA

          Each of these polynucleotide mixtures was labeled at its 3' end with TAMRA dye (Molecular Probes, Eugene, OR). TAMRA dye has an absorption maximum at 565 nm and an emission maximum at 580 nm; its molecular extinction coefficient is 89,000.

25                   The glass slide was prepared for attachment of the polynucleotides of the array by generating isothiocyanate groups (-N=C-S) on the surface of the slide. The slide was derivatized with isothiocyanate groups according to the following protocol:

- 40                   (1)    Soak glass slide in 1 M HCl for 16 hr. (Alternatively, soak in 1 M nitric acid for 3 hr.) Rinse thoroughly with deionized water, followed by acetone. Allow to air dry.
- 30                   (2)    Soak slide in hexane, acetone, and methanol, respectively, for 10 min each. Air dry when done. The slide must be completely dry before proceeding to the next step.
- 45                   (3)    Prepare a solution containing 2% aminopropyltriethoxy silane in 95% acetone:water in a plastic container and let stand 10 min to activate.
- 50                   35

Submerge slide in this silane solution for about 2 min and immediately rinse with acetone. Wash slide with 3 consecutive acetone washes. Allow to completely air dry.

(4) Cure slides by baking in a dry incubator at 98 C for 45 min. Remove from incubator and allow to cool for at least 10 min.

(5) Dissolve 1,4-phenylene diisothiocyanate (PDC) in a 10% solution of pyridine:dimethyl formamide to yield a final concentration of 0.2% PDC. Submerge the slide in the PDC solution and incubate for 2 hr at room temperature. Remove the slide and wash by submerging in methanol for 5 min, followed by two successive baths of acetone for 5 min each. Allow slide to air dry.

For each spot of the array, small volumes of polynucleotides mixtures containing 50  $\mu$ M of the particular degenerate polynucleotide pool for that spot and 0.04  $\mu$ M of the labeled polynucleotide mix (0.08% of the total concentration) were prepared. These mixtures were then spotted onto the prepared slide using a robotic pin spotting device. The spotted polynucleotides covalently bonded to the surface of the slide through a bond between the cyanate molecule on the slide and the 5' amine of the polynucleotide.

Before assaying the array, the baseline level of fluorescence at each location in the array was established by scanning the array to detect the amount of labeled tracer polynucleotide at each spot (FIG. 1).

The array was then assayed with a 241 base, single-stranded target DNA derived from exon 7 of the p53 gene and selected sets of TAMRA dye-labeled 5-base polynucleotides. After allowing the target DNA, attached polynucleotides, and labeled polynucleotides to hybridize under conditions intended to discriminate between perfect matches and single-base mismatches, ligase was added. The ligase covalently joined labeled polynucleotides to attached polynucleotides in the spots of the array at which they bound the target DNA in adjacent positions. The arrays were then washed to remove the target DNA and unligated labeled polynucleotides. The array was again scanned to determine the total fluorescent signal at each position on the array (FIG. 2).

The intensity signals from each spot on the probed array were then normalized to the baseline signals from the corresponding spot on the array, to account for differences in polynucleotide attachment efficiency. The intensities were normalized according to the formula  $(I_a - I_b)/I_b$ , where  $I_a$  is the assay signal intensity and  $I_b$  is the background signal

5 intensity. The normalized intensities were then used to identify mutations in the p53 gene by discriminating between perfect complements and mismatches.

10 As a control, the same assay was also performed on an array generated by the same protocol as the labeled array, but without the use of the labeled polynucleotide tracer mixtures. Because it lacked the tracer in the spots of the array, the intensities could not be normalized for the attachment of the polynucleotides.

15 When the results from the two assays were compared, the signals from the array with the tracer (which could be normalized for the efficiency of attachment at each spot) led to the detection of the mutation in the p53 gene, while the signals from the array without the tracer (which could not be normalized for the efficiency of attachment at each spot) could not.

20 The present invention is not to be limited in scope by the exemplified embodiments which are intended as illustrations of single aspects of the invention. The foregoing specification and accompanying drawings is considered to be sufficient to enable one skilled in the art to broadly practice the invention. Indeed, various modifications of the above-described means for carrying out the invention which are obvious to those skilled in the relevant arts are intended to be within the scope of the following claims. All patents, patents applications, and publications cited herein are hereby incorporated by reference in their entireties for all purposes.



## Claims

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WHAT IS CLAIMED IS:

1. A spatially-addressable array of immobilized molecules comprising a substrate  
10 having immobilized thereon at least two unique compounds, the structures of which are  
5 identifiable by their spatial addresses, wherein each address in the array further comprises  
an amount of a detectable label which is proportional to the amount of molecule  
immobilized at that address.
- 15 2. The array of Claim 1 which is a one-dimensional array.
- 10 3. The array of Claim 2 in which the substrate is a solid-phase synthesis support, glass  
20 fiber or capillary tube.
- 15 4. The array of Claim 1 which is a two-dimensional array.
- 25 5. The array of Claim 1 in which the substrate is a glass or plastic sheet.
- 30 6. The array of Claim 1, in which the immobilized molecule is a polynucleotide.
- 35 7. The array of Claim 6 in which the polynucleotide is covalently attached to the  
25 substrate, optionally by way of a linker.
- 40 8. The array of Claim 7, in which the polynucleotide is covalently attached via a  
terminal nucleotide.
- 45 9. The array of Claim 1 which comprises 10 to 10<sup>6</sup> unique compounds.
- 50 10. The array of Claim 7 in which each immobilized polynucleotide is independently 6  
30 to 20 nucleotides in length.
- 55 11. The array of Claim 10, in which each immobilized polynucleotide is 6-20  
nucleotides in length.
12. The array of Claim 11, which comprises the complete set of polynucleotides 6-10  
35 nucleotides in length.

5 13. The array of Claim 6 in which at least one spatial address has immobilized thereto a mixture of polynucleotides.

10 14. The array of Claim 13, in which the mixture is of the formula:  $N_x B_y N_z$ , wherein N represents any of the four bases and varies for the polynucleotides in a given mixtures, B represents any of the four bases but is the same for each of the polynucleotides in a given mixture, and x, y, and z are integers.

15 15. The array of Claim 14, in which each spatial address has immobilized thereto a mixture of polynucleotides.

20 16. The array of Claim 1 in which the detectable label is a fluorophore.

25 17. The array of Claim 6 in which the detectable label is a fluorescently-labeled polynucleotide.

18. The array of Claim 17 in which the fluorescently-labeled polynucleotide at each address has the same primary sequence as the polynucleotide immobilized at that spot.

30 19. The array of Claim 17 in which the fluorescent label is TAMRA.

35 20. The array of Claim 17, in which the same fluorescently-labeled polynucleotide is immobilized at each address.

40 21. The array of Claim 20, in which the fluorescently-labeled polynucleotide comprises a mixture of fluorescently-labeled polynucleotides.

5 22. The array of Claim 21, in which the mixture of fluorescently labeled polynucleotides comprises:

10 NNNGGCAT-F,  
5 NNCCGGAG-F,  
NNNAACTG-F,  
NNNATGAA-F,  
15 NNNTGTAC-F,  
10 NNNACTGG-F,  
NNNGAACC-F,  
20 NNNTACAG-F,  
NNNCTGGA-F, and  
15 NNCCCGGA-F,

25 wherein N represents any of the four bases and F is a fluorophore.

23. The array of Claim 22, in which F is TAMRA.

30 24. A method of making a spatially-addressable array of compounds, comprising the steps of:

(i) immobilizing at a first address of a substrate a first reagent comprising a first molecule and a first tracer moiety; and

35 (i) immobilizing at a second address of a substrate a second reagent comprising a second molecule and a second tracer moiety.  
25

40 25. In a method of making a spatially addressable array of polynucleotides by depositing pre-synthesized polynucleotides at discrete spatial addresses, the improvement comprising immobilizing an amount of a tracer moiety at each spatial address that is proportional to the amount of polynucleotide immobilized at that address.  
30

45 26. In a method of making a spatially addressable array of compounds by *in situ* synthesis, the improvement comprising immobilizing at each spatial address of the array an amount of a tracer moiety that is proportional to the amount of a product of the *in situ* synthesis immobilized at that address.  
50 35

5 27. A method of increasing discrimination in an array assay comprising:  
contacting an array according to Claim 1 with a molecule that is capable of  
generating a signal upon interacting with an element of the array; and  
10 normalizing the signal intensities from each address of the array.

5 28. A method of increasing the ability to discriminate between perfect matches and one  
base mismatches in hybrids between probe polynucleotides and a target polynucleotide  
15 comprising:

contacting an array of probe polynucleotides according to Claim 6 with the target  
10 polynucleotide under conditions which distinguish between perfectly matched hybrids and  
hybrids containing single base mismatches, wherein said target polynucleotide generates a  
20 signal upon hybridization to any of said probe polynucleotides; and  
normalizing the signal intensities from each address of the array.

15

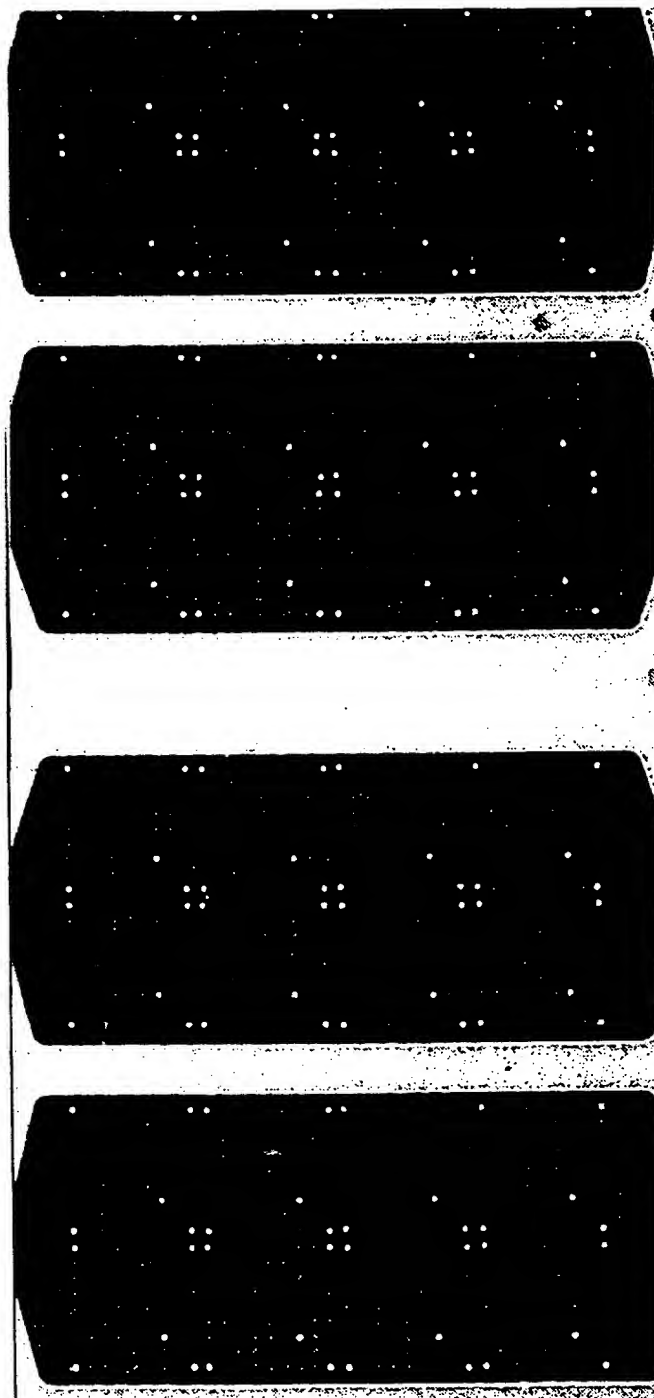
20

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30

35

FIG.1



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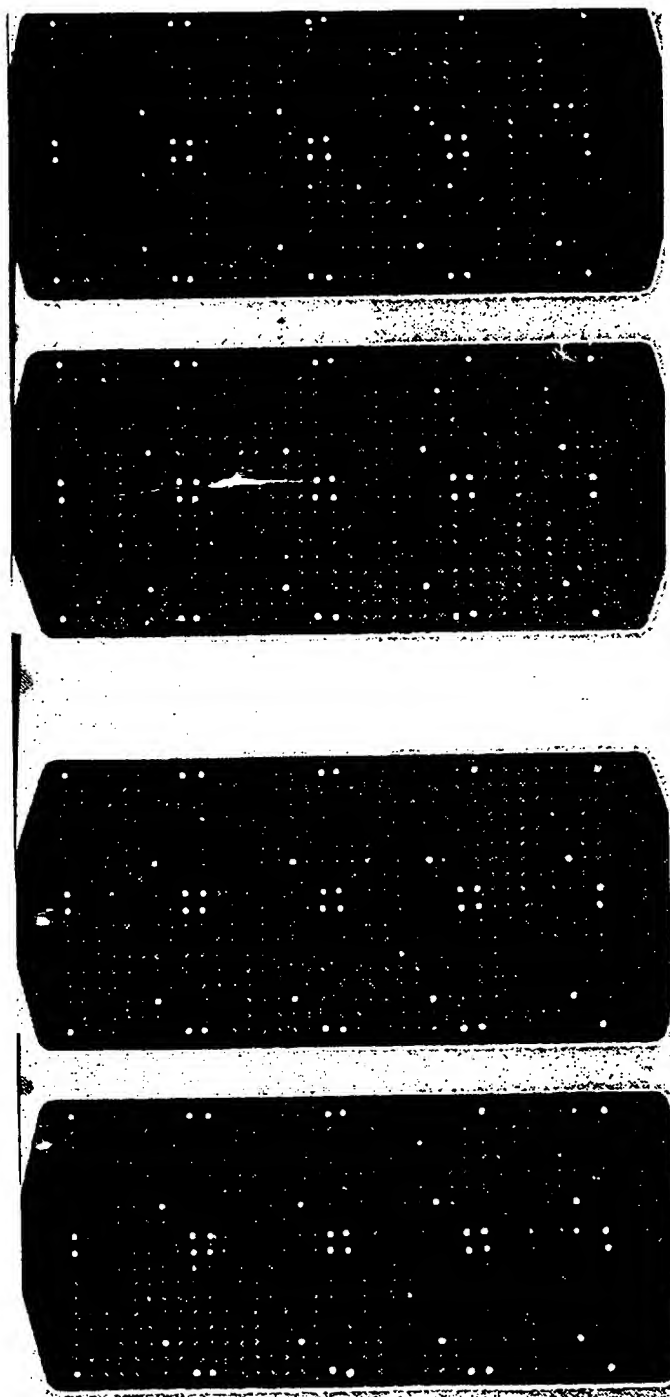


FIG.2

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/29194

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/68  
US CL : 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
U.S. : 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
CAPLUS BIOSIS DEGENE MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,445,934 A (FODOR et al) 29 August 1995 (29.08.1995), see entire document,	1-21, 23-28
Y	US 5,723,591 A (LIVAK et al), 03 March 1998 (02.03.1998), column 4, lines 34-67, column 3, lines 1-60,	1-28
Y	US 5,822,472 A (DANIELZIK et al) 13 October 1998 (13.10.1998), column 10, lines 24-48,	1-28

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is considered to be of particular relevance

"E" earlier application or -ness published on or after the international filing date

"L" documents which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" documents referring to oral disclosure, use, exhibition or other means

"P" documents published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand a principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, each combination being obvious to a person skilled in the art

"A" document member of the same patent family

Date of the actual completion of the international search

08 March 2000 (08.03.2000)

Date of mailing of the international search report

17 APR 2000

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703)305-3230

Authorized officer

Jeffrey S. Lundgren

Telephone No. (703) 308-0196

JOYCE BRIDGERS  
PARALEGAL SPECIALIST  
CHEMICAL MATRIX